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cDNA-SRAP ANALYSIS OF DIFFERENTIAL GENE EXPRESSION IN FOXTAIL MILLET (*Setaria italica* L.*)* UNDER DROUGHT STRESS

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ABSTRACT

Foxtail millet (Setaria italica L.) is an important food and fodder grain crop in arid and semi-arid regions of Asia and Africa. Being an elite drought-tolerant crop, foxtail millet is thought to be an excellent experimental model in studying abiotic stress tolerance system. The aim of this research was to demonstrate the feasibility of cDNA-SRAP for differential gene expression in Setaria italica L. cDNA-SRAP technique, for the first time, was applied in the analysis of differential gene expression in foxtail millet i.e. Setaria italica L. under drought stress. Analysis on the expression profiling of genes revealed that the expression of genes was upregulated by water stress treatment, suggesting that they may involve in the drought tolerance of the crop. This study constitutes the first report that opens up the application of cDNA-SRAP in finding new gene involved in drought tolerance in Setaria italica. Further efforts in validation of identified genes could be explored in development of tolerant lines by MAS as a functional marker and can also be used as candidate genes for development of water stress tolerant transgenics in other related crops.

KEYWORDS: Differential Expression, cDNA-SRAP, Setaria italica & Abiotic Stress

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Original Article

INDRODUCTION

Foxtail millet (*Setaria italica* L. Beauv) is one of the oldest cultivated millet crops serving as food grain in Asia and as forage/fodder in America, Australia and Africa ranking second in the world's total production of millets after pearl millet. India ranks second after China in the world in small millet production with Tamil Nadu and Andhra Pradesh as the leading producers. It is an important food and fodder grain crop in arid and semi-arid regions of Asia and Africa. Being an elite drought-tolerant crop and the attributes like small genome (1 C ~ 515 Mb; $2n = 2x = 18$), low amount of repetitive DNA, a highly conserved genome structure relative to the ancestral grass lineage, inbreeding nature and short life cycle, makes foxtail millet an excellent experimental model in studying abiotic stress tolerance system (Devos et al., 1998; Jayaraman et al., 2008; Doust et al., 2009; Li and Brutnell, 2011; Zhang et al., 2012, Lata et al., 2013).

The adaptation of foxtail millet to low water conditions has been ascribed to its relatively small leaf area, the cell arrangement in its epidermis, its thick cell walls, and its ability to form a dense root system (Li, 1997). Also, the water use efficiency of foxtail millet has been shown to be higher than that of maize, wheat, and sorghum (Gu et al., 1987). However, the molecular mechanism underneath of its drought adaptation is still not clear. Understanding drought tolerance at molecular level particularly identification of relevant genes in foxtail millet is likely to pave the ways for mitigating the drought stress losses in major crops. An extensive germplasm collection of the crop is available, providing opportunities to study the various biological processes and to fetch out the

molecular mechanism underneath its tolerance.

The biological differences among the genotypes used, plant growth conditions, stress treatment conditions and their detection methodologies may result in variation in extent of stress adaptive mechanism. The tolerant species may express some novel stress responsive genes. Hence, comparison of gene expression profiles between contrasting genotypes can provide much information in understanding the spatial and temporal patterns of gene expression required for abiotic stress tolerance**.** The identification, manipulation and comprehension of gene expression patterns would play an important part in unlocking the mysteries of drought responses and adaptation. First report about analysis of differentially expressed transcripts (early- and late- induced) in foxtail millet cv. Prasad after dehydration stress was given by Lata and colleagues (2010). The previously reported as well as unknown genes suggests their function in possible regulation of dehydration adaptation in this crop (Lata et al., 2010). Various unknown genes in response to abiotic stress have been reported in foxtail millet. Zhang et al. (2012) identified 586 genes that were predicted to have roles in stress responses. Qi et al. (2013) analyzed the whole transcriptome of foxtail millet by using the next generation deep sequencing technology and identified a total of 2,824 genes with drought-responsive expression patterns (Qi et al., 2013). Differentially expressed signaling pathways of up-regulated genes in foxtail millet were studied and significantly up-regulated genes were identified in Yugu-1 in response to rust in foxtail millet (Li et al., 2015). To gain a better understanding of the molecular responses of this crop to dehydration stress, various methods based on transcript profiling can be used for the analysis for differentially expressed genes. Sequence-related amplified polymorphism (SRAP) is one such PCR-based molecular marker technique developed by Li and Quiros in 2001. This technique has the advantages of being simple, effective, and fast. Analysis and detection of the fragment length polymorphism with SRAP primers can be carried out with either genomic DNA (SRAP) or cDNA (cDNA-SRAP) as template.

So far, SRAP has also been successfully applied to research of several crops including cotton, melon, buffalograss, peach, and squash with application in various areas including genetic map construction (Li and Quiros, 2001, Lin et al., 2005], molecular diversity analysis (Ferriol, 2003, Kosman and Leonard, 2007) and comparative genomic study (Li et al., 2003). Lu and Wu (2006) carried out a differential display study on salt-tolerance of *Spartina angelica* using cDNA-SRAP in which a differentially expressed fragment was identified (Lu and Wu, 2006). Deng et al. (2007) applied cDNA-SRAP in the study of differential gene expression in the restore and maintainer lines of cabbage. Ma et al. (2008) analyzed differential genes related to seed-coat color in *Brassica napus* L. by cDNA-SRAP technique. Zhang and Nakajima (2015) explored and screened SRAP markers associated with main salt tolerance gene in maize while Huang et al. (2015) identified smut-responsive genes in sugarcane using cDNA-SRAP. In the present study, cDNA-SRAP technique, for the first time, was used to screen differentially expressed genes related to drought-tolerance in *Setaria italica*. Semi-quantitative evaluation of the relative mRNA accumulation can show upregulation/downregulation of genes involved in stress tolerance. the genes identified here for the tolerance mechanism of foxtail millet can be explored in development of tolerant lines by MAS as a functional marker and can also be used as candidate genes for development of water stress tolerant transgenics in other related crops. This study presents the first report of the feasibility of cDNA SRAP for revealing the molecular mechanism of drought tolerance and also opens up its application in differential gene expression in *Setaria italica*.

MATERIALS AND METHODS

Plant Material

The experimental material of the present investigation comprised of foxtail millet (*Setaria italica* L.) accessions procured from National Bureau of Plant Genetic Resources, Regional station, Akola. Two contrasting foxtail millet accession IC97189 (tolerant) and IC97109 (susceptible) (collectively based on the physiological and biochemical screening) (Gawai et al, 2013) were planted in triplicate in pots (20 litre capacity) separately. Pots were watered normally (once per day) until the plants attain 21day period. The 21-day-old seedlings were subjected to water with-holding experiments for a period of 9 days. Water treatment served as the control designated as unstressed (US). Seedlings were sampled by harvesting and freezing immediately in liquid nitrogen and stored at -80˚C for RNA isolation.

Total RNA Extraction

Total RNA was isolated using TRIzol (Invitrogen, USA) using a manufacturer's protocol with minor modifications and the concentration was determined using nanophotometer (IMPLEN, Germany). Following extraction, the isolated total RNA was electrophoresed in 1.5% denaturing agarose gel to check for RNA quality and integrity. First strand cDNA were generated and equalized for further amplification studies.

cDNA-SRAP (Sequence Related Amplified Polymorphism Marker Profiling)

A set of SRAP markers developed by Li and Quiros (2001) were utilized which consisted of five forward and six reverse primers (Table 1). The forward primer set is designed with GC-rich core sequence which targets exonic region in the genome; however the reverse primer set is designed to contain AT-rich core sequence which targets promoter and intronic region of gene/genome. Each primer contains a random filter sequence at the 5'end and three variable selective nucleotides at the 3' end. The reaction was performed by adding following components in order to sterile thin-walled PCR tubes for each PCR amplification reaction: 12.6µl of RNase-free water, 2µl of 10X PCR Buffer, 1.2µl of 50mM $MgSO_4$, 2.0µl of dNTP mix (2mM each dNTP), 0.5µl of upstream primer (0.1 µg/µl), 0.5µl of downstream primer (0.1 µg/µl), 1µl of experimental first-strand cDNA reaction, 0.2µl of *Taq polymerase* (5U/ul). The PCR reaction conditions were, initial denaturation at 94ºC for 5 minutes, 5 cycles of denaturation at 94ºC for 1 minute, annealing at 35ºC for 1 minute and extension at 72ºC for 1 minute, followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute and extension at 72ºC for 1 minute final extension at 72ºC for 5 minutes and reaction was hold at 4ºC. All the PCR products were stored at 4ºC until resolved on 2% agarose prepared in 1x TBE buffer containing 0.5 µg/ml ethidium bromide (EtBr). The amplified products of the cDNA-SRAP were concentrated to about 10 μL with the centrifugal vacuum concentrator (Eppendorf, Germany). The whole volume was loaded onto the 2% agarose gel (containing EtBr) for electrophoresis and imaged with a syngene Gel Imaging System.

Table 1: SRAP Primer Sequences used during the Present Investigation

SN	SRAP Primer	Sequence	Tm
		Forward Primers	
1	me1,	5'-TGAGTCCAAACCGGATA-3',	50°C
2	me2,	5'-TGAGTCCAAACCGGAGC-3',	50°C
3	me3,	5'-TGAGTCCAAACCGGAAT-3',	50°C
4	me4,	5'-TGAGTCCAAACCGGACC-3',	50°C
5	me5,	5'-TGAGTCCAAACCGGAAG-3'	50°C
		Reverse Primers	
1	em1,	5'-GACTGCGTACGAATTAAT-3',	50°C

Table 1: Contd.,			
2	em2,	5'-GACTGCGTACGAATTTGC-3',	50°C
3	em3	5'-GACTGCGTACGAATTGAC-3',	50°C
4	em4,	5'-GACTGCGTACGAATTTGA-3',	50°C
5	em5	5'-GACTGCGTACGAATTAAC-3',	50°C
6	em6,	5'-GACTGCGTACGAATTGCA-3'.	50°C

Scoring Amplicons

Differential analysis on the basis of number of amplicons (present/absent) as well as differences in amplicon intensities to understand differential expression pattern in stressed and unstressed of tolerant and susceptible foxtail millet accessions was done. For TDFs those were expressed differentially with change in amplicons intensities were analyzed by densitometric analysis. This analysis based on pixel intensities of bands produced intensity derived values (IDVs) in AlphaEaseFC (Genetic technologies Inc.) image processing software. Generated values were used to compare intensity of gene expression in further studies.

RESULTS AND DISCUSSIONS

Even though the drought-tolerance capacity of foxtail millet is ascribed to its cellular and morphological characteristics (Li, 1997), there have been very less study on differentially expressed genes that impart drought tolerance to foxtail millet. Differential gene expression pattern was studied by using random primers like cDNA SRAP. Strikingly, the number of genes that were significantly upregulated under drought stress were much higher in tolerant than in susceptible accession.

The initial step in expression studies is isolation of good quality RNA. In this regard, RNA was extracted from 21 day old seedling from the respective plant type using trizol method and checked for quality and quantity on 1.5% denaturing agarose gel. Agarose gel electrophoresis and alpha imager analysis revealed that RNA extracted in this study can meet the quality requirement for cDNA-SRAP analysis. Amplification products presented in all cDNA-SRAP reactions indicated that the reaction conditions adopted here were suitable for the analysis of cDNA-SRAP differential display in foxtail millet. There were neither primer-dimers nor the occurrence of non-specific amplifications. Accurate normalization of template is an important step in gene expression studies. Hence, to confirm the normalisation of cDNA, appropriate internal control gene like α tubulin and EF1α were used which showed constant expression in unstressed and stressed condition. EF-1 α was used in the present study which was suggested as reliable internal control gene in foxtail millet gene expression studies (Kumar et al., 2013). Semi-quantitative RT-PCR has emerged as a versatile technique in transcriptomics, as it can generate rapid measurement of mRNA levels in minimal tissue samples. This study also revealed that PCR product concentrated by the centrifugal vacuum system to increase its concentration, which was helpful to obtain prominant band in agarose gel electrophoresis, while electrophoresis of PCR products without concentration showed rather weak bands.

In cDNA-SRAP technique, the core sequence of the forward primer (CCGG) and the reverse primer (AATT) and the changing annealing temperature ensure the stability of the amplification results (Li et al, 2003; Lu and Wu, 2006; Deng et al., 2007; Ma et al., 2008). The forward and reverse primers can combine with each other freely; hence a series of primer combinations can be achieved. One can also replace the three selective bases at the 3' ends of the forward and reverse primers to develop more primer combinations and can reduce the cost of the primer synthesis. cDNA-SRAP mainly amplifies the open reading frame (ORF) of the genome, hence can increase the correlation between amplicon and

phenotype. Compared with the other differential expression study techniques, cDNA-SRAP is simple and has better repeatability which makes it more reliable and economic. The cDNA-SRAP technique has been proved to be suitable for analysis of differential gene expression in several kinds of plants discussed earlier (Li et al., 2003; Lu and Wu, 2006; Deng et al., 2007; Ma et al., 2008).

Out of the 30 primer combinations of SRAP, only 12 primer combinations showed good amplification of which 4 combinations were monomorphic and 8 combinations were polymorphic. Semi-quantitative evaluation of the relative mRNA accumulation showed upregulation/downregulation in the various primer combinations studied. Nine of the twelve primer combination showed upregulated expression after treatment, two combinations F1-R5 and F4-R5 showed neither upregulated nor downregulated expression and combination F2-R1 showed downregulation in expression of the gene amplified in stressed condition. The number of amplicons produced by an individual primer combination was ranged from one (F4-R1) to eleven (F6-R4) with an average of 5.66 amplicons per primer combination i.e. Combination F6-R4 showed amplification of maximum genes whereas combination F4-R1 showed amplification of single gene (Figure 1). Expression pattern of possibly water stress-related genes through cDNA-SRAP is presented in the form of heatmap (Figure 2). The amplicons obtained in each primer combination were designated as 1-68 in continuous manner. Primer combination F2-R5 and F1-R1 showed significant high expression in tolerant accession. Amplicon number 49, 50, 51, 54, 55, 56, 60, 61, 62, 63, 65, 66, 67 showed consistent expressions in both contrasting accessions in unstressed and stressed conditions.

CONCLUSIONS

In conclusion, the SRAP marker system is a simple and efficient marker system to study differential gene expression studies. The study provides a basic research for the application of cDNA-SRAP analysis for understanding new putative transcripts and/or genes that are differentially expressed in *Setaria italica* in response to water stress. It has several advantages over other systems: simplicity, reasonable throughput rate, discloses numerous co-dominant markers, allows easy isolation of bands for sequencing and, most importantly, it targets ORFs. cDNA-SRAP can also be adapted for a variety of purposes in different crops, including map construction, gene tagging, genomic and cDNA fingerprinting, and mapbased cloning. Water withholding experiments in plants can simulate a series of physiological and biochemical reactions leading to expression of genes related to drought-tolerance. The high expression of many genes under stress condition confirms their role in drought tolerance. Further validation of these genes could help in identifying the potential candidate genes involved in drought tolerance. Differentially expressed new putative transcripts and/or genes can be a useful tool for understanding their function in response to stress.

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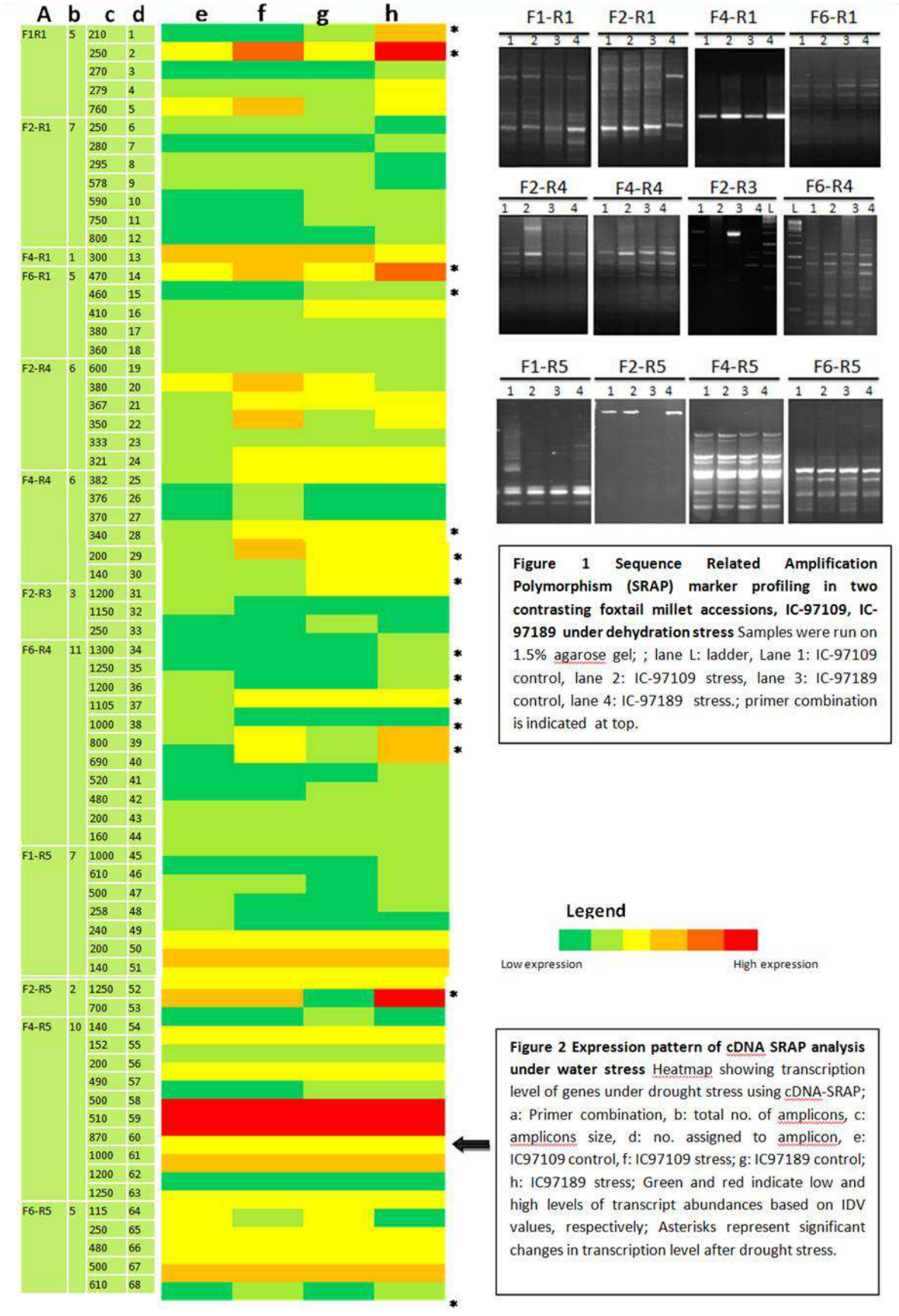

Figure 1 Sequence Related Amplification Polymorphism (SRAP) marker profiling in two contrasting foxtail millet accessions, IC-97109, IC-97189 under dehydration stress Samples were run on 1.5% agarose gel; ; lane L: ladder, Lane 1: IC-97109 control, lane 2: IC-97109 stress, lane 3: IC-97189 control, lane 4: IC-97189 stress.; primer combination is indicated at top.

Figure 2 Expression pattern of cDNA SRAP analysis under water stress Heatmap showing transcription level of genes under drought stress using cDNA-SRAP; a: Primer combination, b: total no. of amplicons, c: amplicons size, d: no. assigned to amplicon, e: IC97109 control, f: IC97109 stress; g: IC97189 control; h: IC97189 stress; Green and red indicate low and high levels of transcript abundances based on IDV values, respectively; Asterisks represent significant changes in transcription level after drought stress.